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HPLC-DAD Validation Method for Quantifying Gallic Acid in the Aerial Parts of *Schinus terebinthifolius*

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Schinus terebinthifolius Raddi (Anacardiaceae), commonly known as "*pimenta rosa*", "*aroeira da praia*", "*aroeira*", "*aroeirinha*", and "*cabuí*" is frequently used in traditional medicine to address various conditions, including skin wounds, ulcers, tumors, diarrhea, arthritis, as well as urinary and respiratory tract infections. This study aimed to develop and validate an analytical method using high-performance liquid chromatography with diode array detector (HPLC-DAD) for quantifying gallic acid (GA) in methanolic extracts of *S. terebinthifolius* leaves, branches, and fruits. The chromatographic separations utilized a reversed-phase Zorbax Eclipse XDB-C18 column with an isocratic elution mode of acetonitrile/methanol/water acidified with 0.2% formic acid. The flow rate was 0.8 mL min^{-1}, and detection occurred at 254 nm. The developed HPLC-DAD method demonstrated selectivity, with a GA retention time of 5.263 min and absorption maxima at 220 and 271 nm. The method exhibited high linearity (coefficient of 0.9996), precision (relative standard deviation (RSD) values < 5%), and robustness. Accuracy ranged from 100.28 to 111.71%, with an average recovery of 105.41% and an average RSD of 3.46%. These validated results play a crucial role in assessing and standardizing raw materials containing *S. terebinthifolius*, emphasizing the reliability of the developed HPLC-DAD method for GA quantification in different plant parts.

Keywords: pink pepper, gallic acid, analytical, Anacardiaceae

Introduction

Schinus terebinthifolius Raddi (Anacardiaceae) is native to South America and commonly known as "*pimenta rosa*", "*aroeira da praia*", "*aroeira*", "*aroeirinha*", and "*cabuí*".[1] It is popularly employed for the treatment of various conditions such as skin wounds and ulcers, tumors, diarrhea, arthritis, urinary and respiratory tract infections,[2,3] gout, serving as an antiseptic, anti-inflammatory, balsamic, and hemostatic agent.[4] Additionally, it is used for addressing heart problems, inflammation, and providing hepatoprotective effects.[2,5]

Studies[3,4,6,7] indicate that *S. terebinthifolius* is a plant rich in secondary metabolites including phenols, tannins, flavonoids, anthraquinones, and essential oils. These compounds contribute to its anti-inflammatory, antimicrobial, antiviral, and medicinal properties, which are beneficial for cognitive, neurodegenerative, and metabolic disorders, as well as conditions such as diabetes, obesity, hypertension, and hypercholesterolemia. Tannins, in particular, play a significant role in these properties.[8] Tannins are classified as hydrolysable and condensed, and they accumulate in almost all parts of dicotyledonous plants. Hydrolysable tannins are characterized by a central polyol whose hydroxyls are esterified with gallic acid (GA). Therefore, the concentration of this class can be determined by quantifying GA, which is released during acid hydrolysis.[9,10] GA has been identified in *S. terebinthifolius*[1] in various parts of the plant, including the bark,[9,11] fruits,[12,13] and leaves.[14]

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GA is a benzoic acid of significant importance in the synthesis of hydrolysable tannins. It is widely distributed across various plant families, including Anacardiaceae, Fabaceae, and Myrtaceae.[15,16] Scientific studies have revealed several beneficial properties associated with GA,[17] including antioxidant effects, anticancer properties,[18] anti-HIV activity,[19] antiulcerogenic potential,[20] anti-inflammatory attributes,[21] as well as antimicrobial, and antifungal qualities.[22,23]

Enhancing the quality control of herbal products relies significantly on the precise identification of a chemical marker. This marker consists of a clearly defined constituent or groups of constituents that are inherently present in the plant product. While there is some research on the biological activities and chemical constitution of *S. terebinthifolius*, the development of simple and validated methods to quantify marker compounds is necessary to enhance quality control. Although various analytical techniques can be employed to identify and quantify these substances, high-performance liquid chromatography (HPLC) is the most used method.[24-26]

For this reason, this study aims to develop and validate an analytical method using high-performance liquid chromatography-diode array detector (HPLC-DAD) for quantifying GA in the methanolic extract of leaves, branches, and fruits of *S. terebinthifolius*.

Experimental

Botanical material

Samples of leaves, branches, and fruits from *S. terebinthifolius* were collected in Goiânia, GO, Brazil, (786 m, 16º53'59" south, and 49º13'29" west), at Praça Universitária, Setor Leste Universitário, in October 2020, during the morning period. A specimen meticulously was prepared, identified and deposited in the Herbarium of the Federal University of Goiás under the reference number UFG-66444. Subsequently, samples of leaves, branches, and fruits were subjected to drying in an air circulation oven (INOVA model 171, Brazil) at a temperature of 38 ± 2 ºC. The dried samples were then ground into a powder form using Poli® industrial blender (model LS-08MBR-N, Brazil).

Reagents, solvents, and standards

Ethanol 96º GL (Neon, Suzano, São Paulo, Brazil), HPLC grade methanol (MeOH, J.T. Baker, Mexico), HPLC grade acetonitrile (J.T. Baker, Mexico), formic acid (Organics, New Jersey, USA), and gallic acid (GA; VETEC, Duque de Caxias, Rio de Janeiro, Brazil) were used.

Obtaining the crude ethanol extract (CEE)

The previously pulverized material underwent a maceration process at room temperature (± 28 ºC) for three days, with intermittent stirring using 96° GL PA ethanol as the extracting solvent. The ratio employed was one part of the pulverized material to 1:5 g mL^{-1} of ethanol (100 g in 600 mL^{-1}). Following maceration, filtration was performed using a funnel and filter paper, and the resulting extract was concentrated in a rotary evaporator at a temperature of 40 °C. The plant residue underwent two additional extraction cycles following a similar procedure to the initial one, ultimately yielding the ethanolic extract from the leaves of *S. terebinthifolius* (CEE).[27]

Development and validation of the method for quantification of GA by HPLC

The samples for validation, consisting of leaves from *S. terebinthifolius* that were sprayed and dried, were weighed in triplicates, with 5 g of leaves for every 10 mL of methanol. The maceration process was facilitated by ultrasound (Q5.9/40A, 40 kHz, Ultronique, São Paulo, Brazil) for 15 min. The resulting methanolic extract underwent filtration through filter paper and a 0.45 µm polyvinylidene fluoride (PVDF) membrane (Millex®, Massachusetts, USA). The methanol extract was subsequently diluted at a 1:1 v/v ratio with MeOH to decrease its concentration before injection into the chromatograph system.

To initiate the method development, a combination of acetonitrile and water acidified with 0.2% formic acid was employed. Subsequently, varying proportions of methanol were introduced and tested at different flow rates ranging from 0.8 to 1.0 mL min^{-1} and different temperatures. The objective was to identify a more cost-effective method with reduced processing time, improved substance separation, and adherence to the parameters outlined in the RDC No. 166/2017.[28]

The analyses were conducted using a Waters Chromatographic System model HPLC, Alliance (Massachusetts, USA), featuring an e2695 separation module, 2998 diode array detector (DAD), and Empower 2.0[29] data processing system. Chromatographic separations were performed on a reversed-phase Zorbax Eclipse XDB-C18 (USA) column (250 mm × 4.6 mm, 5 µm) LN-B14036. The mobile phase consisted of a mixture of HPLC analytical grade acetonitrile (pump A), HPLC analytical grade MeOH (pump B), and ultrapure water (Milli-Q) acidified with 0.2% formic acid (pump D). The mobile phase composition was 8% (A), 2% (B), and 90% (D), utilizing an isocratic elution mode with a flow rate of 0.8 mL min^{-1} for 14 min, and detection at 254 nm.

The injection volume was 10 µL. Analyzes were conducted at a temperature of 24 °C. The mobile phase was previously filtered through a 0.45 µm polyvinylidene fluoride (PVDF) membrane (Millex, Massachusetts, USA) and degassed using an ultrasonic bath (Q5.9/40A, 40 kHz, Ultronique, Brazil).

System suitability

Before conducting the validation, the chromatographic system utilized for the analysis underwent an evaluation to ensure its capability to produce consistent and reproducible results. This assessment was carried out through system suitability compliance experiments, defined as a series of tests to ascertain that the equipment can generate results of acceptable accuracy and precision. The parameters, in accordance with the Food and Drug Administration (FDA)[30] and Ribani *et al.*[31] include (*i*) retention factor (k): $k > 2$; (*ii*) repeatability-relative standard deviation (RSD) < 1% for $n > 5$; (*iii*) resolution (Rs): $Rs > 2$ between the peak of interest and the nearest potential interferent (impurity, degradation product, or other substances); (*iv*) tail factor (TF): ≤ 2; (*v*) the number of column theoretical plates (N): generally should be > 2000 for HPLC.

Validation of the analytical method

Validation was conducted in accordance with the guidelines outlined in Resolution No. 166/2017 of the National Health Surveillance Agency (ANVISA) for Category I, specifically addressing quantitative tests for the potency of the active ingredient in pharmaceutical products or raw materials.[28]

Selectivity

The method's selectivity was assessed by identifying GA in the sample through a comparison of the retention times and ultraviolet absorption spectra (190 to 400 nm) of the peaks obtained in both the sample and the reference standard GA. Additionally, chromatograms and absorption spectra of the HPLC analytical grade methanol diluent were examined to detect potential interfering peaks in the analyses.

Linearity and interval

For the construction of the standard curve, seven solutions with GA concentrations 10, 50, 100, 200, 300, and 400 µg mL^{-1} were prepared using HPLC analytical grade methanol. The standard solutions were filtered through a 0.45 and/or 0.22 µm Millex® membrane and then injected, in triplicate, into the chromatograph. The average areas for each marker concentration were plotted on the ordinate axis, with the corresponding concentrations on the abscissas. The equation of the line was obtained by the method of least squares, according to the equation 1.

$$y = a + bx \quad (1)$$

where a: inclination of the line to the axis; b: intersection of the line with the y-axis.

The curve was generated using Microsoft Excel 2013.[32] The test results were processed using Statistica 7[33] software, which included conducting regression significance tests via analysis of variance (ANOVA) and assessing the normality of the residuals using the Anderson-Darling method. All calculations were performed with a 95% confidence interval.

Limits of detection and quantification

The limits of detection and quantification were calculated with equations 2 and 3, respectively:

$$LOD = DPa \times 3\ IC \quad (2)$$
$$LOQ = DPa \times 10\ IC \quad (3)$$

where LOD: limit of detection; LOQ: limit of quantitation, DPa: standard deviation of the intercept with the Y axis of the calibration curve; IC: slope of the analytical curve.

Precision (repeatability and intermediate accuracy)

For precision evaluation, both repeatability (intra-day precision) and intermediate precision (inter-day precision) were determined. Precision was evaluated by determining the concentration at three points on the analytical curve: the low level (3000 µg mL^{-1}), medium level (7000 µg mL^{-1}), and high level (11000 µg mL^{-1}) for repeatability. The low, medium, and high levels correspond to 80, 100, and 120%, respectively.

The solutions were filtered through a 0.22 µm Millex® membrane (Massachusetts, USA) and injected (in triplicate) into the chromatograph. Intermediate precision was conducted by a different analyst on a separate day, with sample preparation following the same conditions mentioned above. The coefficient of variation (CV), was calculated using the Microsoft Excel 2016[32] program to establish compliance with the RDC parameters.[31]

Accuracy

Accuracy was verified by incorporating known amounts

(concentration equivalent to 100 μg mL^{-1}) of the standard GA into the sample solutions at three distinct concentration levels. The accuracy value, expressed as a percentage, was determined by establishing the relationship between the concentration of the standard added to the sample and the concentration of the standard before the addition, following equation 4.

$$\text{Accurancy} = \frac{100 \times [(\text{sample} + \text{standard}) - (\text{standard})]}{(\text{standard})} \quad (4)$$

Robustness

Robustness was assessed by varying the temperature from 24 °C to 22 and 26 °C, adjusting the flow rate from 0.8 mL min^{-1} to 0.7, 0.9, and 1.0 mL min^{-1}, modifying the mobile phase pH from 3.3 to 2.7 and 3.8, and employing another column, Xterra MS C18 (4.6 mm × 250 mm, 5 μm) from the USA. Additionally, a modification was made to the mobile phase with pump A (5%), pump B (2%), and pump C (93%). The CV was calculated by comparing the peak areas of GA at each alteration with the area of the original method.

Matrix effect

Matrix effects were evaluated using the standard additions method. The calibration curve was employed, as described for the evaluation of the linearity of the GA standard (10, 50, 100, 200, 300, 400 μg mL^{-1}) in solvent MeOH. Additionally, the calibration curve of the extract in five levels (30, 50, 70, 90, 110 μg mL^{-1}) was generated with the addition of the standard (100 μg mL^{-1}) (1:1). Each level was prepared in three independent repetitions, which were subsequently analyzed in random order. The parallelism of the straight lines serves as another indication of the absence of interference from the constituents of the matrix, and its confirmation was conducted through appropriate statistical evaluation. Thus, the slopes of both curves were compared using the *t*-test,[34] in accordance with RDC 166/17.[28]

Linearity of the extract

The profile of markers in the complex matrix was examined to assess whether their behavior is linear. Consequently, a linearity analysis of the methanol extract was carried out at concentrations of 30, 50, 70, 90, and 110 μg mL^{-1} in triplicate on the chromatograph, and the analytical curve was constructed. The mean areas for each concentration of GA were plotted to derive the equation of the straight line using the least squares method.

Quantification of GA in leaves, branches, and fruits

Leaves, branches, and fruits were extracted in triplicate (5 g in 10 mL^{-1}) with MeOH using an ultrasound device at room temperature for 15 min. The peak areas corresponding to GA were collected and analyzed.

Statistical analysis

All validation data were added to Microsoft Excel[32] spreadsheets. Linearity and matrix effect analysis were performed using the *t*-test in the statistical program PAST 4.06[35] and homoscedasticity using Microsoft Excel and PAST 4.06.

Results and Discussion

In earlier investigations, the polar extract of *S. terebinthifolius* leaves was subjected to phytochemical analysis by Santana *et al.*[36] leading to the isolation of GA derivatives and glycosylated flavonoids. Ceruks *et al.*[37] isolated five phenolic compounds, including GA, from fractions obtained from the ethanolic extract of *S. terebinthifolius* leaves. Glória *et al.*[13] through HPLC, identified two phenolic compounds in *S. terebinthifolius* Raddi fruits: naringenin and GA. Feuereisen *et al.*[38] utilized ultra-high performance liquid chromatography tandem mass spectroscopy (UHPLC-DAD-MS/MS) analysis to identify four anthocyanins, three bioflavonoids, GA, and two types of hydrolysable tannins in the exocarp extract of *S. terebinthifolius* fruits. Additionally, Migues *et al.*[39] identified polyphenolics such as catechin, GA, epicatechin, rutin, quercetin, and chlorogenic acid in *S. terebinthifolius* bark extracts through HPLC-DAD.

Development of a method for quantification of GA by high-performance liquid chromatography (HPLC-DAD)

An isocratic method was developed with the mobile phase consisting of 8% (A) and 2% (B), 90% (D) and a flow rate of 0.8 mL min^{-1} for 7 min, and detection at 254 nm was found to be the most suitable based on parameters according to the U.S. Food and Drug Administration (FDA)[30] and Ribani *et al.*[31]

The method selection aimed to ensure precise and rapid analysis. Consequently, the GA standard eluted at a retention time of 5.448 min.

Regarding temperature variation, it was observed that at 24 ºC, peak resolution improved. The optimal balance between retention time and peak resolution was

achieved with a flow rate of 0.8 mL min^{-1}. Subsequently, the acetonitrile/methanol/acidified water gradient mobile phase at a flow rate of 0.8 mL min^{-1} demonstrated the best separation of GA substances in the complex matrix, with a retention time of 5.448 min. These chromatographic conditions were found to be within the system control parameters for pure GA peak and in complex matrices, including TF, Rs, retention factor, and the N in accordance with FDA[30] and Ribani *et al.*[31] (Table 1).

Table 1. Parameters of compliance with the system suitability according to FDA[30] and Ribani *et al.*[31] of standards (GA) and methanol extract of *Schinus terebinthifolius* leaves

	GA		Parameters (FDA)[30]
Retention time (t_R) / min	5.263	5.448	
Parameters	standard	extract	
Retention factor (k)	4.95	4.90	≥ 2
Resolution (Rs)	4.47	2.04	≥ 2
Tail factor (TF)	1.00	1.08	≤ 2
Number of theoretical plates	4.71×10^3	4.80×10^3	> 2

GA: gallic acid.

Within the parameters established by the FDA,[30] the developed isocratic method showed t_R, Rs, and the number of theoretical plates greater than two, as well as a TF less than two. The results indicate that the method conditions are suitable for the quantification of the GA marker, even in the complex matrix, in this case, the plant drug, *S. terebinthifolius* leaves. Compared with the method validated by Carvalho *et al.*,[9] it demonstrated a shorter run time and reduced use of organic solvents, contributing to the establishment of a more sustainable method.

Validation of the analytical method for the quantification of GA by HPLC-DAD

Selectivity, linearity, and interval

The chromatographic profile and UV spectrum of GA (400 μg mL^{-1}) in MeOH were obtained through HPLC-DAD analysis. GA presented a retention time of 5.263 min (Figure 1a) with maximum absorption of 220.5 and 271.4 nm (Figure 1b). The sample extract in MeOH (7 g 10 mL^{-1}) showed a retention time for GA of 5.448 min (λ_{max} 220.5 and 271.4 nm). These chromatographic profiles did not reveal interfering substances in the GA retention time. Moreover, the UV spectrum of the samples was considered identical to the standard, demonstrating the selectivity of the method.

The GA calibration curve (Figure 2) exhibited a linear response within the range of 10-400 μg mL^{-1}, and the linear prescription for GA was $y = 6808.4x + 6071.8$. The analytical curve demonstrated an impressive linearity of 0.9996, indicating a strong fit of the data to the regression line. This underscores that the obtained results are directly proportional to the concentration of the analyte in the sample.

Linearity data were further assessed using the ANOVA test, revealing that the calculated *F* value for the model surpassed the tabulated *F* value for a 95% confidence level. This demonstration indicates that the model was suitable for predicting the data.

The homoscedasticity of the data for the two markers was examined using the Cochran test. For GA, the calculated C was 0.5036 which is less than the critical C of 0.616. Therefore, the null hypothesis was accepted, and the adopted data were considered homoscedastic. The significance of the angular coefficient was assessed using the *F* ANOVA test, revealing that the calculated *F* value

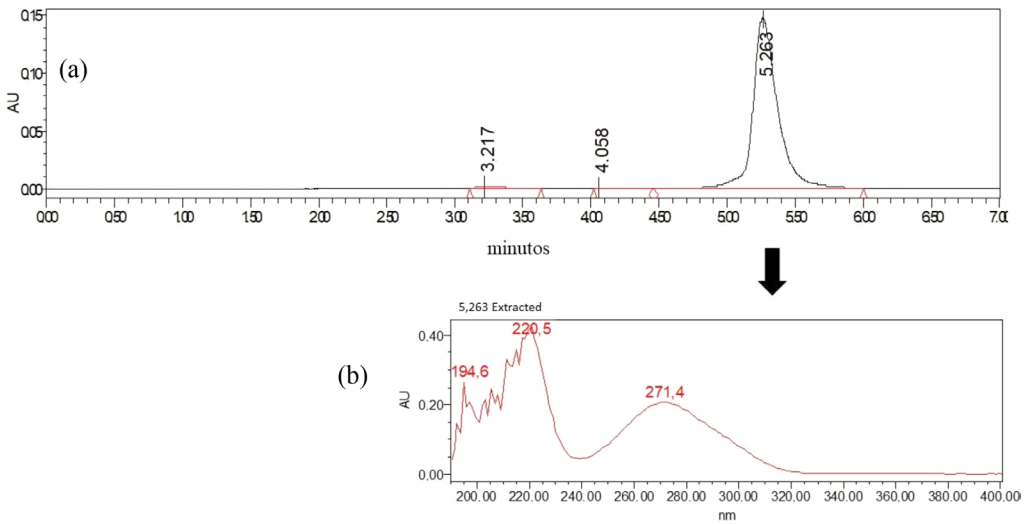

Figure 1. Chromatogram of the GA standard (a) and UV spectrum (b) obtained using Empower 2.0 Program.[29]

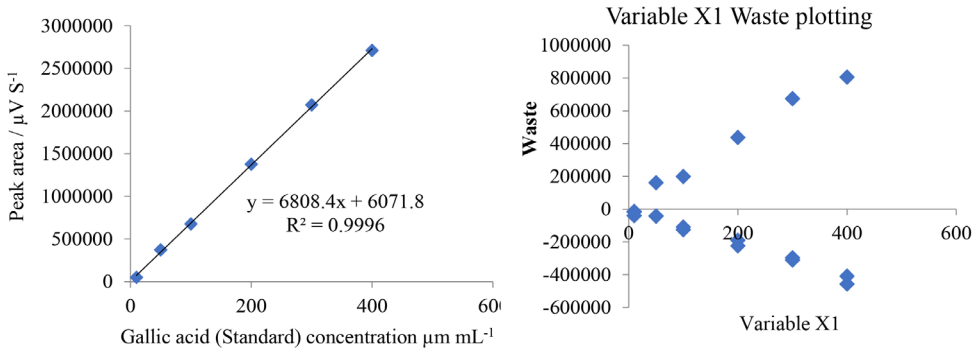

Figure 2. GA analytical curve (a) in the concentration range of 10-400 µg mL^{-1} and plot of GA residues (b). GA: gallic acid.

of 125.6 exceeded the critical *F* value of 4.49 for GA. As a result, the null hypothesis was rejected, suggesting that the peak area (*y*) varies with the concentration of the analytes (*x*), demonstrating the linearity of the method. The angular coefficient was also examined using the Student's *t*-test, and the calculated *T* value for GA (11.20) exceeded the critical *T* value (2.447). Therefore, the null hypothesis was rejected, providing evidence that the angular coefficient is statistically different from zero.[28]

Limits of detection and quantification

The LOD value for GA was determined to be 3.57 µg mL^{-1}, representing the smallest detectable amount of the analyte in the sample, although not necessarily quantifiable. Concerning the LOQ value, it was determined to be 10.82 µg mL^{-1}, representing the lowest measurable concentration of the analyte in the sample using the proposed method.[31] The experiments were conducted within a range above the limits, and therefore, the concentration values obtained for GA were deemed satisfactory.

In the literature, Carvalho *et al.*[9] obtained an LOD of 3.12 ng mL^{-1} and an LOQ of 25 ng mL^{-1} while analyzing the aqueous ethanolic extract of the bark of *S. terebinthifolius*. Silva *et al.*[40] observed an LOD of 1.6 µg mL^{-1} and an LOQ of 5.1 µg mL^{-1} for GA in the ethanolic extract of the leaves of *Eugenia punicifolia* L.

Precision

Regarding method precision (Table 2), RSD values were below 5% for triplicates of low, medium, and high concentrations, as recommended by the specifications.[27] Precision at the repeatability level indicates the correlation between method results executed under the same conditions within a period. Intermediate precision suggests that, even with different analyzes on different days, the method can yield consistent results.[41]

Accuracy

In terms of accuracy (Table 3), the method yielded recoveries ranging from 100.28 to 111.71% with a mean of 105.41% and mean RSD of 3.46%. These data align with the acceptable recovery intervals for tests on complex matrices (80-120%) such as natural products.[42] The recovery test quantifies the amount of analyte added to the test material that is extracted and subject to quantification.[43]

Robustness

Regarding robustness, variations in column temperature, pH, flow, method, and column, resulted in RSD values below 5%, except for the flow of 0.7 and 1.00 mL min^{-1}, for both the peak area and GA content (Table 4). This demonstrates the method's ability to remain consistent with the tested variations, and it contributes to the transferability of the analytical process to other laboratories.[44]

The robustness tests revealed that variations in the flow rate ratio (0.7 and 1.0 mL min^{-1}) of the mobile phase influenced peak area and retention time with an RSD greater than 5%. However, other performance data suggested the reliability of this method.

Matrix effect

The matrix effect is a selectivity study that investigates possible interference caused by compounds that make up the sample matrix, potentially leading to phenomena such as a decrease or increase in signal or instrumental response.[45] The matrix effect was evaluated following RDC 166/17,[28] and the confirmation of parallel lines was conducted through appropriate statistical evaluation. The confirmation that the lines are parallel indicates the absence of interference from matrix constituents. The *t*-test was employed using the statistical program PAST 4.06.[35] All regression assumptions were confirmed for the solvent-matrix combination calibration curves. The matrix effect was not significant concerning the slopes of the solvent and matrix curves for the GA within the studied ranges ($p > 0.05$). The *p*-value for GA in the parallelism test (Table 5) was 0.1. As they are greater than 0.05, the

Table 2. Precision data of the HPLC analytical method at repeatability levels and intermediate precision for the quantification (%) of GA in the methanol extract of *S. terebinthifolius* leaves (λ = 254 nm)

Method linear range concentration level	GA content / (mg mL^{-1})	Area / (μV S^{-1})	GA / %
	121.3929	832,563	40.464
Low 80% (3.000 μg mL^{-1})	122.6352	841,021	40.878
	121.8527	835,694	40.617
	312.9944	2,137,063	44.713
Medium 100% (7.000 μg mL^{-1})	294.3341	2,010,016	42.047
	314.3426	2,140,211	44.906
	449.3019	3,065,099	40.845
High 120% (11.000 μg mL^{-1})	493.7627	3,367,806	44.887
	477.4354	3,256,643	43.403
RSD / %			4.58
Intermediate precision			
	125.8644	863,007	41.955
Low 80% (3.000 μg mL^{-1})	127.5096	874,208	42.503
	127.3332	873,007	42.444
	312.5297	2,133,899	44.647
Medium 100% (7.000 μg mL^{-1})	311.3225	2,125,680	44.475
	331.6887	2,264,341	47.384
	456.7947	3,116,113	41.527
High 120% (11.000 μg mL^{-1})	475.0708	3,240,544	43.188
	469.3126	3,201,340	42.665
RSD / %			4.18

RSD: relative standard deviation; GA: gallic acid.

Table 3. Accuracy data of the HPLC analytical method for the quantification of GA in methanolic extract of *S. terebinthifolius* leaves (λ = 254 nm)

Method linear range concentration level	GA area in the sample	GA area in sample + GA pattern / (μV S^{-1})	GA recovery area	GA concentration / (mg mL^{-1})	GA recovery / %
	832,563	2,268,203	3,703,843	543.119	103.200
Low (3000 μg mL^{-1})	841,021	2,245,642	3,650,263	535.249	101.704
	835,694	2,401,011	3,966,328	581.672	110.525
	2,137,063	2,878,562	3,620,061	530.813	100.861
Medium (7000 μg mL^{-1})	2,010,016	2,804,650	3,599,284	527.762	100.281
	2,140,211	2,972,799	3,805,387	558.033	106.034
	3,065,099	3,485,050	3,905,001	572.665	108.814
High (11000 μg mL^{-1})	3,367,806	3,579,413	3,791,020	555.923	105.633
	3,256,643	3,632,854	4,009,065	587.949	111.718
Theoretical concentration of the GA standard					526.28
Recovery average (RSD) / %					105.41

RSD: relative standard deviation; GA: gallic acid.

hypothesis that the angular coefficients are equal at a significance level of 5% is not rejected. In this case, the lines are considered parallel.

Linearity of GA in the complex matrix

A pure substance within a pre-established range must exhibit linear behavior. The linearity of the GA marker (Figure 3) was verified, demonstrating that even in the complex matrix, which is the extract, the compound maintains a linear character with a correlation coefficient equal to 0.9968 for GA under the same conditions as those used for testing the analytical curve of the standard.

Table 4. Robustness of the method considering the variation in column temperature, flow, pH, method, and column compared to the original method developed (λ = 254 nm)

Gallic acid (GA)						
Condition		Area / (μV S^{-1})	Average	Average between parameters	SD	RSD / %
Original method developed		2,170,110		–	–	–
		2,199,166	2,205,486	–	–	–
		2,247,182		–	–	–
Temperature / °C	22	2,181,156				
		2,241,936	2,211,971	2,208,728	4,585,6	0.20
		2,212,821				
	26	2,206,241				
		2,318,361	2,250,883	2,228,184	32,101	1.44
		2,228,049				
pH	2.7	2,201,340				
		2,202,678	2,215,052	2,210,269	67,647	0.30
		2,241,140				
	3.8	2,230,081				
		2,374,628	2,251,835	2,228,660	32,774	1.47
		2,1507,97				
Flow / (mL min^{-1})	0.7	2,646,852				
		2,511,313	2,661,931	2,433,708	322,756	13.26
		2,827,630				
	0.9	2,306,604				
		2,060,863	2,233,236	2,219,361	19,622	0.88
		2,332,242				
	1.0	2,289,512				
		1,872,074	2,018,551	2,112,018	132,183	6.25
		1,894,067				
Method	A (5%), B (2%), C (93%)	2,008,216				
		2,445,231	2,185,827	2,195,656	13,901	0.63
		2,104,036				
Column	Xterra MS C 18 250 × 4.6 5 μm	2,263,884	2,326,102	2,269,036	80,703	3.55

SD: standard deviation; RSD: relative standard deviation; GA: gallic acid.

Table 5. Angular coefficient of the standard in solvent and the fortified extract and the result of the parallelism test for GA using the statistical program PAST 4.06

	Gallic acid (GA)
Angular coefficient with matrix	316,934
Angular coefficient without matrix	292,112
p-value	0.1
F-test	0.1

Dosage of GA in the extract of leaves, branches, and fruits

The chromatographic profile of the aerial parts of *S. terebinthifolius* was conducted using HPLC-DAD (Figure 4) and revealed the presence of the GA peak in the leaves (5.448 min), branches (5.186 min), and fruits (5.164 min). All exhibited UV signals characteristic of GA with maximum absorption at 220 and 271 nm.

The gallic acid content in the methanolic extract of the leaves, branches and fruits of *S. terebinthifolius* is 7 μg mL^{-1}. The methanolic extract of the leaves extracted by ultrasound had the highest GA content at 44.71%, followed by the methanolic extract of the branches at 31.53%, and the methanolic extract of the fruits at 4.87%.

Carvalho *et al.*[9] developed and validated an HPLC method to quantify GA in the bark of *S. terebinthifolius*, collected in the Atlantic Forest in Paraíba-Brazil. The extraction was performed by maceration (5 days) with occasional agitation using ethanol/water (4:7). The method utilized an acetonitrile/water gradient, starting with 15 to 80% over 20 min, with a flow rate of 1.0 mL min^{-1}. The study demonstrated that the hydroalcoholic extract of *S. terebinthifolius* bark at a concentration of 10.0 μg mL^{-1} (based on the dry residue of the plant) yielded a concentration of 24.5 ng mL^{-1}. Compared to Carvalho *et al.*[9] the method developed and validated in this study for quantifying GA in *S. terebinthifolius* is faster in both extraction and analysis

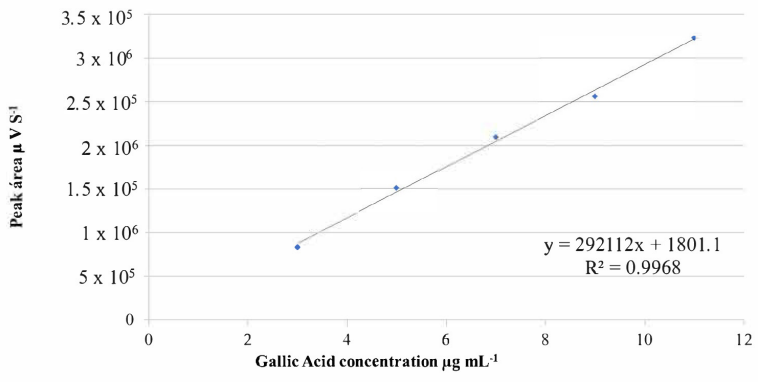

Figure 3. Linearity of GA in the complex matrix, ethanol extract of *S. terebinthifolius*.

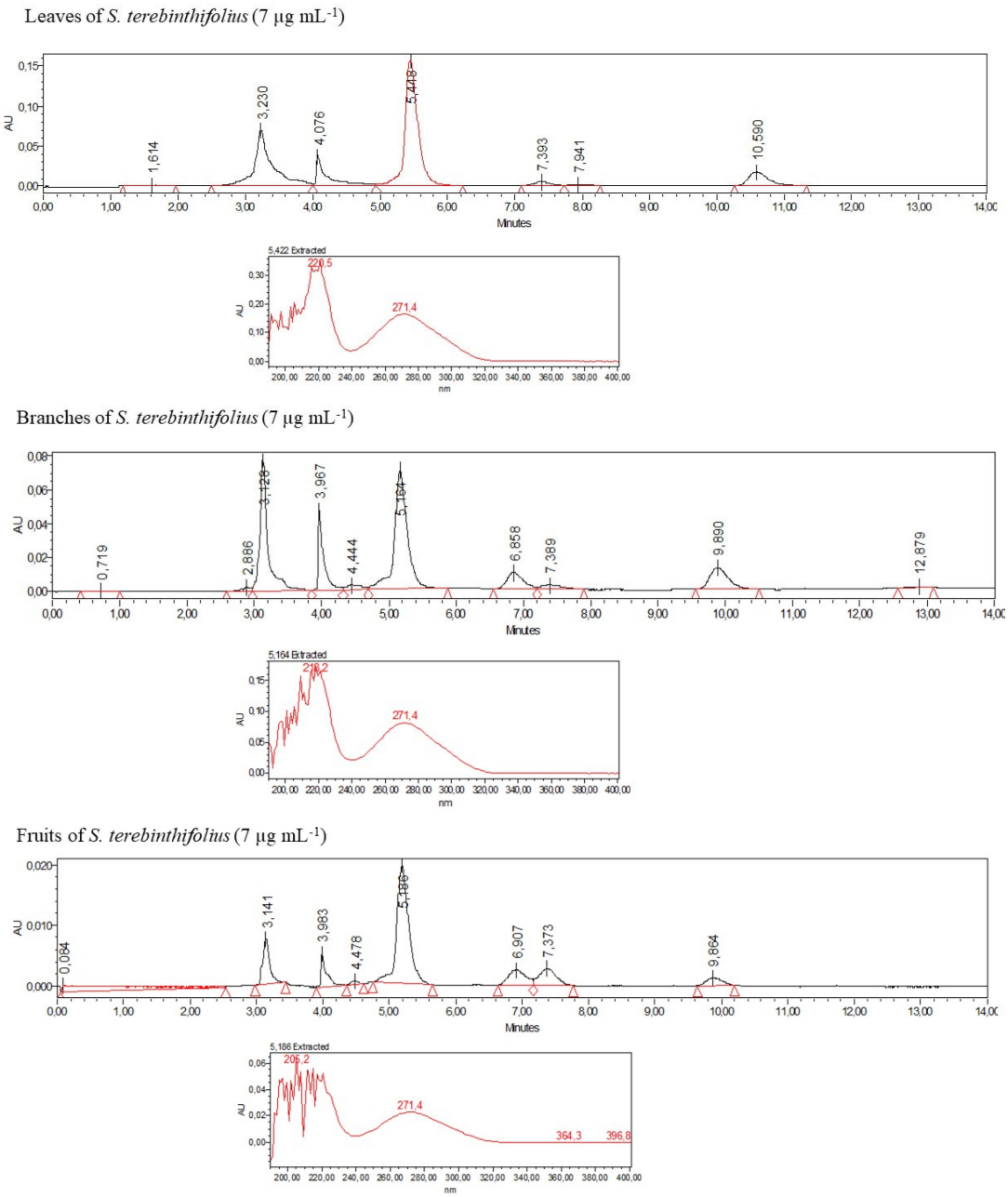

Figure 4. Chromatographic profile of the methanolic extract of the leaves, branches, and fruits of *S. terebinthifolius* and ultraviolet spectrum at 254 nm.

times. The compounds were extracted using a modern method (ultrasonography for 20 min) while the other involved a conventional method, maceration for 5 days.

Migues *et al*.[39] developed and validated the HPLC-DAD method for identifying and quantifying polyphenols, including GA, in the extract of *S. terebinthifolius* bark collected in different regions of Brazil. The method utilized a gradient with water acidified with phosphoric acid and acetonitrile for 120 min at 35 ºC. After analysis, a concentration of 3.2 to 4.8 mg mL^{-1} of GA was found in samples of 10.0 mg L^{-1}. Silva *et al*.[40] developed and validated an HPLC-DAD method to quantify GA in *Eugenia punicifolia* leaves; however, the method involved a gradient, with a higher consumption of acetonitrile and an analysis time of 20 min.

The method developed and validated in this work is simpler because it is isocratic and involves less consumption of organic solvents, adhering to the principles of green chemistry. Additionally, it is faster in both the extraction of compounds and the analysis time in HPLC-DAD. The developed method allowed for better quantification of GA in leaves, branches, and fruits, enabling the inference of hydrolysable tannin concentrations. Therefore, this method facilitates the quantification of a marker responsible for ensuring the quality control of plant or herbal material from the aerial parts of *S. terebinthifolius*.

Conclusions

The chromatographic analyses validated in HPLC-DAD, quantified hydrolyzable tannins as GA in the methanolic extracts of leaves, branches, and fruits of *S. terebinthifolius*, which allowed the development of a method to obtain quality control of the plant material, also indicating variations in the chemical composition of the aerial parts. This methodology is suitable for quality control of herbal medicine, as the conditions were optimized for the HPLC-DAD system.

Chromatographic separation was evaluated using parameters such as t_R, Rs, TF, and the number of theoretical plates in accordance with the FDA. The selected wavelength provided good sensitivity for GA, and detection by DAD was adequate, showing low limits of detection. The parameters were observed, and the method proved to be selective, linear, precise, accurate, robust, and without matrix effects, making it suitable for the technical evaluation and treatment of chromatographic data obtained for the analysis of hydrolysable tannins in extracts of *S. terebinthifolius.*

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Author Contributions

Christiane F. Martins was responsible for conceptualization, data curation, formal analysis, investigation, methodology, writing (review and editing, original draft); Liliane S. Silva for conceptualization, data curation, formal analysis, investigation, methodology, writing (review and editing, original draft); Emannuel I. A. Campos for review and editing; Leonardo L. Borges for statistical analysis; Tatiana S. Fiuza for writing-review and editing, José Realino de Paula for conceptualization, funding acquisition, investigation, project administration, resources, supervision, writing (original draft, review and editing).

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